THE PURIFICATION AND CHARACTERISATION OF PENICILLIN V

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A large number of penicillins, differing from one another solely in the nature of the amide side chain, have been reported in the past. One, penicillin V (phenoxymethylpenicillin), has long been known to be elaborated by strains of *Penicillium notatum* when cultured in the presence of suitable precursors[1]. It has recently been shown that penicillin V is resistant to inactivation by acids, and can be isolated as the free acid which is sparingly soluble in water[2]. Penicillin V has been used clinically in place of penicillin G (benzylpenicillin) preparations[3,4]. As the lability of penicillin G at low *p*H necessitates its oral application in special formulations of the soluble salts or as sparingly soluble salts, it seems that penicillin V will be particularly suitable for oral use.

Methods for the rapid assay of penicillin V, were needed in the course of purifying a sample of this material for use as a reference standard for chemical and biological assays. Suitable chemical or physical assay methods were sought and at the same time, methods were devised for the rapid differentiation of penicillin V from penicillin G.

Experimental

General Methods. Ultra-violet absorption spectra were determined in 1 cm. quartz cells in a model SP.500 Unicam Spectrophotometer.

Brominations were made by treating a neutral aqueous solution contained in a suitably closed flask with an excess of solutions of both N potassium bromate and N potassium bromide, and then making acid with a slight excess of concentrated hydrochloric acid. Bromine uptake was determined by the addition of excess solid potassium iodide, and titration with sodium thiosulphate solution.

Purification of Penicillin V

The initial work was done with a sample of penicillin V supplied by "Biochemie" Gesellschaft M.B.H., Kundl, Innsbruck, Austria. This material (sample A) contained no volatile matter, and appeared to be pure when assayed iodimetrically[13]. However, chromatographic assay[14] showed that it contained a small amount of a second biologically active component which remained at or near the point of application. Attempts to separate the two components were followed by means of iodimetric and chromatographic assays. The results are treated in detail elsewhere[13,14], but are described briefly here.

When sample A was dissolved in water by the addition of sodium bicarbonate, and was then precipitated by the slow addition of slightly less than the theoretical quantity of dilute hydrochloric acid, the product (sample B) contained somewhat less of the second component but its

purity, determined iodimetrically, rose to over 100 per cent. Crystallisation of sample B from aqueous acetone yielded a product (sample C) with an even higher purity, and containing only a trace of the second component. Solubility analyses of samples A, B and C by the method of Webb[5] using the spectrophotometric technique[13], suggested that sample C was, and sample A was not, homogeneous.

A quantity of penicillin V (sample D), supplied by the Production Division of this company, was purified by the methods described in the previous paragraph. The product (sample E) when examined chromatographically, and by solubility analysis appeared to be homogeneous. When assayed iodimetrically it appeared to have a purity greater than 100 per cent. Further attempts to purify sample E have failed to increase the iodimetric potency, and the material appears to be pure.

The Ultra-violet Absorption Spectrum of Penicillin V

Penicillin V has been reported[1] to contain two relatively sharp absorption maxima in the ultra-violet band. The spectra of purified penicillin V and of its sodium salt were therefore re-examined.

The free acid (sample E) was dissolved in water. The spectrum was found to contain maxima at 268 mμ and 274 mμ, with a minimum at 272 mμ (Fig. 1). The adsorption at each maximum obeyed Beer's Law in the range 0·00 to 0·04 per cent. w/v. Because solution of the acid is slow unless the sample is finely divided, and decomposition begins on standing for a few hours in aqueous solution, the preparation of solutions of the free acid should not be attempted at concentrations above 0·025 per cent. w/v. The molecular extinction coefficient of penicillin V is 1330 at 268 mμ and 1100 at 274 mμ.

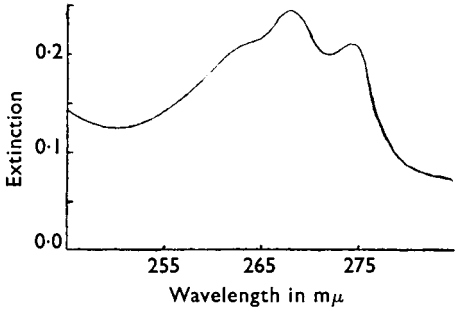

FIG. 1. The ultra-violet absorption spectrum of phenoxymethylpenicillin (0·0067 per cent. w/v in water).

Solutions of penicillin V in chloroform show a spectrum similar to that of aqueous solutions, but the positions of the maxima are shifted slightly to 270 mμ and 276 mμ. Ethanol may also be used as a solvent for the free acid, which may be determined quantitatively from the absorption at 268 mμ and 274 mμ after dilution to 5 per cent. v/v ethanol content.

The sodium salt of penicillin V, which is readily soluble in water, was prepared from the free acid and sodium bicarbonate solution; its spectrum is similar to that of the free acid, with absorption maxima at 268 mμ and 274 mμ. The absorption at each maximum obeyed Beer's Law in the range 0·00 to 0·03 per cent. w/v. Unlike the free acid, solutions of the sodium salt do not decompose on standing. There is no detectable change in the absorption intensities after ten days at room temperature.

The Ultra-violet Absorption Spectra of Alkaline and Acidic Degradation Products

Treatment of penicillin V with 0·5N sodium hydroxide solution at room temperature for 15 to 30 minutes causes complete decomposition of the penicillin V. After neutralisation, examination of the ultra-violet absorption spectrum, with an appropriate blank, reveals that it resembles penicillin V, with peaks at 268 mμ and 274 mμ. The intensity of absorption at each of these wavelengths is approximately the same as that exhibited by an equal concentration of undecomposed penicillin V.

Aqueous solutions of penicillin V are acidic, having *p*H 3–4. Examination of the spectrum of a solution of the free acid over a period of time, reveals the gradual appearance of a rather broad peak at 318 to 320 mμ. This peak first appears after 4 to 6 hours, gradually increasing in intensity to a maximum after 1 to 2 days at room temperature. The intensities of absorption at 268 mμ and 274 mμ are affected by this new peak, so that errors are caused if spectrophotometric estimations of penicillin V are made on aged solutions of the acid.

More drastic acidic treatment of penicillin V, by treatment at *p*H 1 at room temperature for 1 hour, causes an increase in the intensities of absorption at 268 mμ and 274 mμ, although the general shape of the absorption curve is similar to that of penicillin V. The peak at 318 to 320 mμ does not appear in solutions of penicillin V degraded at *p*H 1, even when neutralised after treatment at *p*H 1 for only 5 minutes.

Solubility Analyses of Penicillin V Preparations

The samples A, B, C and E described earlier, were examined for purity by the solubility analysis method of Webb[5]. Each sample was finely ground, and increasing weights were slurried in 100 ml. portions of water. The mixtures were agitated vigorously for 2 hours, filtered and diluted. The absorption at 268 mμ, 272 mμ and 274 mμ was then determined for each solution. The operations from the filtration stage onwards were made as rapidly as possible, in order to reduce to the minimum any effects caused by the decomposition of penicillin V in aqueous solution. The results obtained with samples A and C are shown in Figures 2 and 3. It seems that sample C is pure, and sample A contains more than 1 component. Sample E is similar to sample C, and also appears to be pure.

The Ultra-violet Absorption Spectra of Model Phenoxymethyl Compounds

The use of compounds containing the phenoxymethyl grouping as fermentation precursors, necessitated the examination of their ultra-violet spectra since they might interfere in the spectrophotometric assay of impure penicillin V.

The spectrum of phenoxyacetic acid has been reported previously[6]: it contains a single absorption maximum at 270 mμ in water, ethanol or ether, with a molecular extinction coefficient of approximately 1300. Phenoxyethanol was found to possess a similar spectrum with a single absorption maximum at 270 mμ, but with a much lower molecular extinction coefficient of 330.

Phenoxyacetamide was prepared from phenoxyacetic acid (3g.) by refluxing with 5 ml. of thionyl chloride for 1 hour, cooling and treating carefully with excess of concentrated aqueous ammonia. The amide was filtered, washed and crystallised from hot water. It melted at 98° C. (Fritzsche[7] reports a value of 101° C.). The spectrum of this compound

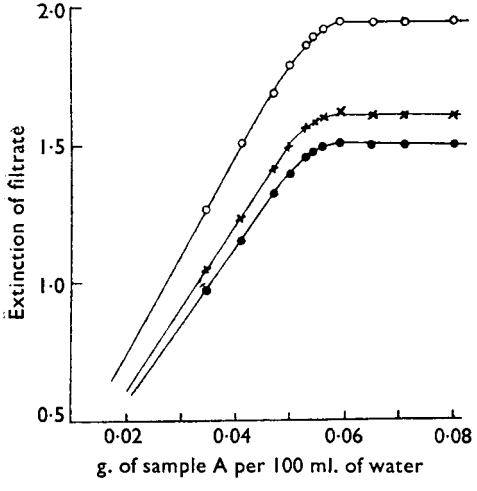

FIG. 2. The solubility analysis of phenoxymethylpenicillin sample A. Extinctions have been corrected for the dilutions made before reading.

○——○ Read at 268 mμ. ×——× Read at 274 mμ. ●——● Read at 272 mμ.

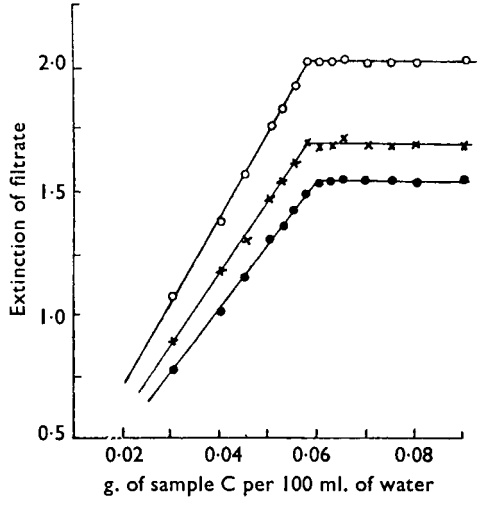

FIG. 3. The solubility analysis of phenoxymethylpenicillin sample C. Extinction have been corrected for the dilutions made before reading.

○——○ Read at 268 mμ. ×——× Read at 274 mμ. ●——● Read at 272 mμ.

had 2 absorption maxima in ethanol solution, at 269 mμ and 276 mμ *N*-Methylphenoxyacetamide was prepared in a similar fashion from the acid chloride and aqueous methylamine solution. After crystallisation from hot water, it melted at 68° C. This compound also exhibited a spectrum with absorption maxima at 269 mμ and 276 mμ in ethanol solution. The values for the molecular extinction coefficients at each peak of both the amide and the methylamide were similar to the values for the corresponding peak of the spectrum of penicillin V.

The Conversion of Pencillin V to Phenoxyacetic Acid

Treatment of penicillin V with boiling 2N sulphuric acid for 2 hours liberates phenoxyacetic acid quantitatively. The intensity of absorption of this acid at 270 mμ obeys Beer's Law in the range 0·00 to 0·01 per cent. w/v, and penicillin V can be determined by extraction of sulphuric acid hydrolysates with ether and measurement of the intensity of absorption of the ethereal extract at 270 mμ.

Attempts were made to assay the phenoxyacetic acid content of penicillin V hydrolysates bromimetrically. The ethereal extracts were washed with sodium hydroxide solution, and the washings neutralised. This

solution was then brominated for 15 minutes at room temperature in the dark, and the bromine uptake determined. Results indicated a bromine absorption of approximately 1 equivalent per mole of phenoxyacetic acid. The scatter between duplicate experiments was, however, too great to use the method for quantitative purposes.

Phenoxyacetic acid did not react with iodine solutions. The presence of this acid in crude penicillin V samples e.g. fermentation broths will thus not interfere with the determination of penicillin V by the iodimetric method[13].

Comparison of the Ultra-violet Spectra of Penicillin V and Penicillin G

The absorption peaks in the spectrum of an aqueous solution of penicillin V can be readily detected at concentrations as low as 0·002 per cent. w/v. Solutions of sodium and potassium penicillins G[8] and of benzathine dipenicillin G show markedly different spectra. At low concentrations, there is an inflection in the spectrum at 250 to 260 mμ: at higher concentrations, this develops into small peaks at 253 mμ and 257 mμ. Procaine benzylpenicillin shows a single, pronounced, absorption maximum at 290 mμ, due to the procaine component of the molecule and detectable at concentrations of about 0·0015 per cent. w/v. The various spectra are collected for comparison in Figure 4.

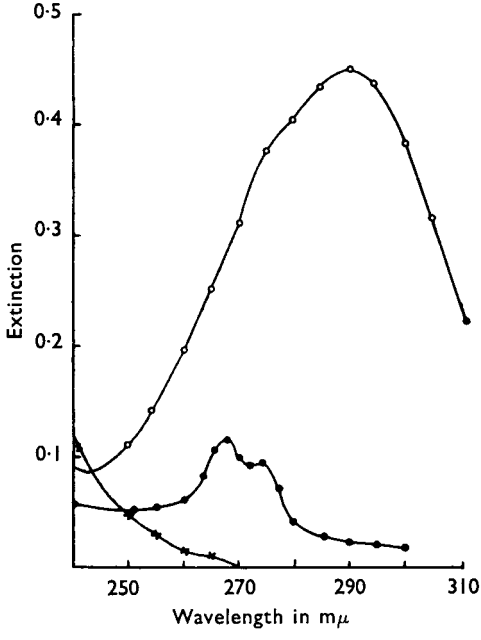

FIG. 4. The ultra-violet absorption spectrum of different penicillin preparations in aqueous solution.
●——● Phenoxymethylpenicillin (0·0023 per cent. w/v).
○——○ Procaine benzylpenicillin (0·0015 per cent. w/v).
×——× Sodium benzylpenicillin (0·0047 per cent. w/v).

Conversion of Penicillin V to p-Bromophenoxyacetic Acid

Early attempts to devise a method for differentiating chemically between penicillin V and penicillin G, were based upon the hope that under suitable conditions, penicillin V could be converted by hydrobromic or hydriodic acids to phenol, for which there are sensitive, specific tests. The initial experiments were made with phenoxyacetic acid. Two grams was refluxed for 2 hours with 20 ml. of 48 per cent. hydrobromic acid. The reaction mixture was cooled and extracted three times with 20 ml. portions of ether.

The bulked extracts were washed twice with 5 ml. portions of N-sodium hydroxide solution, and these alkaline extracts were neutralised. This solution gave only a faint reaction for phenol with the Folin-Ciocalteu reagent. When the phenoxyacetic acid was brominated, a white precipitate separated immediately. Bromination was allowed to proceed for 15 minutes at room temperature in the dark. The precipitate was then filtered, well washed with water, and recrystallised from hot water. After drying, the product melted at 158° C. This product was obviously not 2:4:6-tribromophenol. Further examination showed that it was acidic, with an equivalent weight of 230. It appeared that this material might be *p*-bromophenoxyacetic acid, the initial hydrobromic acid treatment having failed to decompose the phenoxyacetic acid. Confirmation was obtained by preparation of the material from phenoxyacetic acid (1 g. in 150 ml. water), by treatment in the dark at room temperature for 15 minutes with 40 ml. of freshly prepared bromine water. After filtration, washing with water and crystallisation from hot water, the product melted at 158° C., undepressed upon admixture with the previous preparation. Final confirmation was obtained by synthesis from *p*-bromophenol by the method of Koelsch[9]. The product again melted at 158° C., undepressed upon admixture with either of the previous preparations.

These results suggested that penicillin V could be characterised by hydrolysis to phenoxyacetic acid and conversion of this to the sparingly soluble *p*-bromophenoxyacetic acid, for further work, described later, showed that under the conditions employed, phenylacetic acid did not give a sparingly soluble bromo-derivative. Accordingly, the cooled hydrolysate obtained after refluxing 1 g. penicillin V with 20 ml. 2N sulphuric acid for 2 hours, was extracted with ether. The ether extracts were in turn extracted with sodium hydroxide solution, and these extracts were neutralised and brominated in the normal manner. The product was filtered, washed and crystallised from hot water. After drying, it melted at 150° C. Surprisingly, the mixed melting point with authentic *p*-bromophenoxyacetic acid was depressed to 127° C. Examination of the degradation product showed that it contained bromine, but neither nitrogen nor sulphur, and was an acid with an equivalent weight of 220. The ultra-violet absorption spectrum in ethanol solution showed a major absorption maximum at 226 mμ, with a subsidiary peak at 278 to 280 mμ. Authentic *p*-bromophenoxyacetic acid showed a spectrum with peaks at identical wavelengths, but the intensities of absorption at each peak were some 10 per cent. higher than those of the degradation product. It was then found that recrystallisation of this product from a small volume of benzene raised the melting point to 157° C., now undepressed upon admixture with authentic *p*-bromophenoxyacetic acid. The intensities of absorption of the ultra-violet spectral peaks also now practically coincided with those of the synthetic material. The experiment was then repeated, but the crude bromination product was crystallised directly from benzene, to give a product melting at 158° C., undepressed upon admixture with the synthetic material.

Solutions of phenylacetic acid (0·2 g.) in water (20 ml.), when brominated

in the normal manner, gave no precipitate, although some uptake of bromine occurred. Similarly, 2N sulphuric acid hydrolysates of sodium penicillin G, when extracted with ether and brominated as before, also failed to give any insoluble bromo-derivative.

The Colour Reaction of Penicillin V with Chromotropic and Sulphuric Acids

Freed[10] has described a colour reaction for phenylacetic and phenoxyacetic acid derivatives. In this reaction, a small amount of the solid preparation is treated with a few crystals of chromotropic acid and 2 ml. of concentrated sulphuric acid. The mixture is immersed in a glycerol bath at 150° C. for 1 to 2 minutes, and then removed. The colour of the solution is noted, after dilution of the reaction mixture with concentrated sulphuric acid if necessary. The results of treating penicillin V and several penicillin G preparations in this manner is shown in Table I. A number of simpler compounds are also listed. It will be noted that omission of the chromotropic acid from the reaction mixture results in a marked change in the colours produced.

TABLE I

COLOUR DEVELOPMENT IN THE FREED[10] REACTION

Compound	Chromotropic acid plus sulphuric acid	Sulphuric acid alone
Blank	Pale yellow	Colourless
Phenoxyacetic acid	Deep red	Brown
p-Bromophenoxyacetic acid	Deep red	Deep brown
Penicillin V	Deep blue-purple	Orange brown
Phenylacetic acid	Pale yellow	Colourless
Sodium penicillin G	Brown	Light brown
Procaine hydrochloride	Yellow-green	Colourless
Procaine penicillin G	Red-brown	Pale yellow
Benzathine sulphate	Yellow-green	Colourless
Benzathine dipenicillin G	Deep red	Amber
Carboxymethylcellulose	Black	Black

DISCUSSION

The preparation of penicillin V samples having purities apparently greater than 100 per cent. when assayed iodimetrically, is discussed elsewhere[13], where a statistical examination of the results is presented. The purification is proved by the disappearance from chromatograms of the second biologically active component[14], and by the results of the solubility analyses. The alternative explanation, that the purification procedure actually resulted in a concentration of a lower molecular weight penicillin with similar chromatographic and ultra-violet spectral characteristics, is unlikely, in view of the solubility analysis results. Further evidence that purification has occurred, is supplied by the results obtained by Dr. G. H. Twigg (The Distillers Company Limited, Research and Development Department) during an examination of the infra-red spectra of various samples of penicillin V. Sample A was found to possess a spectrum similar to those of samples C and E, but contained in addition 2 rather weak absorption bands at 727 cm.$^{-1}$ and 846 cm.$^{-1}$: these bands occur in the spectral region containing frequencies characteristic of the out-of-plane deformation vibrations of the hydrogen atoms of the benzene

ring, and their absence from the spectra of samples C and E suggests that the second component in sample A differs from penicillin V solely in the nature of the amide side chain. The fact that the second component appears to have ultra-violet spectral characteristics practically identical with those of penicillin V also suggests that the difference in composition is probably very slight.

The ultra-violet spectrophotometric method for the determination of penicillin V is useful for checking the quality of relatively pure samples, but suffers from severe drawbacks when applied to impure materials. The close similarity of the spectra of the penicillin and its acidic and alkaline inactivation products results in inability to detect small amounts of these products in penicillin V. Large quantities of acidic inactivated material will be detectable, but since the molecular extinction coefficient of alkaline inactivated material is close to that of penicillin V, the presence of large amounts of such material will not be noted.

Application of the spectrophotometric method to crude samples, such as fermentation broths and intermediate recovery samples is similarly restricted. Such compounds as phenoxyacetic acid and phenoxyethanol, which may be present and which possess a single maximum at 270 mμ, will prevent use of the method, although the absence of such compounds could be detected from the value of the ratio of the intensities of absorption at 268 mμ and 274 mμ. The presence of the system of 2 absorption maxima at 269 mμ and 276 mμ in such simple compounds as phenoxyacetamide and *N*-methyl-phenoxyacetamide is interesting from the theoretical point of view, but illustrates yet again the unsuitability of the spectrophotometric assay procedure for impure samples.

Samples containing extraneous materials absorbing at the phenoxymethylpenicillin peaks could be assayed for their penicillin content by making use of the formation of the peak at 318 to 320 mμ under mildly acidic conditions. This peak is probably due to the formation of phenoxymethylpenicillenic acid. The formation of the corresponding acids from other penicillins has been used as the basis of a spectrophotometric assay procedure[11,12].

The spectrophotometric assay procedure does enable ready differentiation between penicillin V and penicillin G preparations. Examination of the spectrum of an approximately 0·002 per cent w/v solution will ensure identification.

The estimation of penicillin V by spectrophotometric determination of the phenoxyacetic acid produced on acidic hydrolysis, is of academic interest only, in view of the ease of the direct spectrophotometric method. Moreover, the method suffers from the same drawbacks as the direct method. However, the formation of phenoxyacetic acid can be used for chemically characterising penicillin V, because it readily yields the sparingly soluble *p*-bromophenoxyacetic acid. This is a well defined crystalline compound, which can be further characterised if necessary by means of its ultra-violet spectrum. Under similar experimental conditions, there is no precipitate from brominated hydrolysates of penicillin G preparation.

The reaction of phenoxyacetic acid derivatives, developed by Freed[10]

for the detection of 2:4-dichlorophenoxyacetic acid is sensitive, is rapid to perform, and gives characteristic colours enabling the ready differentiation between penicillin V and various preparations of penicillin G.

Summary

1. The purification of penicillin V has been studied, with the object of obtaining a pure sample for use as a reference standard for chemical and biological assays.

2. A penicillin V preparation has been obtained, which, under the experimental conditions detailed[13], absorbs 2·46 ml. 0·01N iodine solution per mg. after inactivation with penicillinase.

3. Evidence is presented to show that this material is pure.

4. The ultra-violet absorption spectra of penicillin V, its acidic and alkaline decomposition products, and a number of simpler phenoxymethyl compounds, have been determined. Pure penicillin V has a molecular extinction coefficient of 1330 at 268 mμ, and 1100 at 274 mμ.

5. An assay method for penicillin V, based upon the measurement of its absorption in the ultra-violet, is described, and the limitations of the method are discussed.

6. Methods have been found for the rapid differentiation of penicillin V from penicillin G preparations.

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